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DNA Fingerprinting to Improve Data Collection Efficiency and Yield in an Open-Field Host-Specificity Test of a Weed Biological Control Candidate

Brian G. Rector, Alessio De Biase, Massimo Cristofaro, Simona Primerano, Silvia Belvedere, Gloria Antonini, and Rouhollah Sobhian*

An open-field test was conducted in southern France to assess the host-specificity of *Ceratapion basicorne* (Illiger), a candidate for biological control of yellow starthistle. Test plants were infested by naturally occurring populations of *C. basicorne* but were also exposed to sympatric herbivore species, including other *Ceratapion* spp. Insects from the test plants were collected directly into tubes of ethanol and were subsequently identified to species according to DNA sequence similarity with morphologically identified reference specimens. This integrated, morphological and molecular identification method was used in an effort to maximize the amount of data gained in the field bioassay and to minimize the number of taxonomist–hours necessary to complete the study. The results obtained showed that the French *C. basicorne* population only attacked yellow starthistle and cornflower, another known host of *C. basicorne*. Molecular phylogenetic analysis of the insects collected from all other nonhost plants rejected the possibility that any were *C. basicorne*.

Nomenclature: Cornflower, *Centaurea cyanus* L. CENCY; yellow starthistle, *Centaurea solstitialis* L. CENSO; *Ceratapion basicorne* (Illiger).

Key words: Apionidae, biological control, host range, open-field test, safflower.

Host-specificity testing is a major component of candidate assessment for weed biological control (Bellows and Headrick 1999). When testing the host-specificity of insects that feed within target plant tissues, particularly in open-field experiments in which test plants are exposed to many different insect species, identification of all collected insects is crucial to avoiding false-positive results, especially for insects collected from nontarget plants. If only a morphological study of the specimens is performed, such

identification can be difficult and time-consuming and lead to an unacceptable amount of missing data. Adult specimens are usually required for insect identification, and they must be in good physical condition. In addition, an expert taxonomist for the group in question is required, but such a person is not always readily available. Cryptic species or subspecies of the candidate biocontrol agent may also exist with disparate host ranges, and they may be difficult or impossible to separate from the candidate using only morphological comparison (Antonini et al. 2008; Fumanal et al. 2004).

Ceratapion basicorne (Illiger), a weevil that feeds within the root and crown of the noxious weed yellow starthistle (*Centaurea solstitialis* L.; YST), is a biological control candidate for YST in North America (Clement et al. 1989; Smith et al. 2008). It is an apparently univoltine weevil, and the adults emerge from hibernation within the host plant in early spring to feed and mate. Eggs are laid in a host's leaves and hatched larvae mine through the leaf and down the midrib to the root and crown, where they feed and complete their development (Smith and Drew 2006). Evaluation of *C. basicorne* as a biological control candidate has focused on populations originating in eastern Turkey;

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Interpretive Summary

This study demonstrates the application of molecular genetic tools to improve the efficiency and yield of data collection in open-field tests of host-specificity for weed biological-control candidates. The test was conducted in a field where the presence of close relatives of the candidate agent was expected and precise species identification was essential. Use of molecular tools in such experiments enables identification of test insects in immature stages and allows collection of data earlier in the experiment. As such, loss of data due to precocious emergence of adult test insects is avoided. In addition, the number of taxonomist-hours required for the completion of such an open-field study is significantly reduced by the use of molecular tools in the identifications. This is especially important in cases where specialists for the taxon in question are rare. In this study, a French population of *Ceratapion basicorne*, a candidate biocontrol agent of yellow starthistle, was shown to be worthy of further study in the event that alternatives to a more intensively studied Turkish population of the candidate agent are necessary.

these populations have, thus far, been determined to be sufficiently host-specific to merit continued consideration (Smith 2007; Smith and Drew 2006; Smith et al. 2006, 2008; Uygun et al. 2008). However, *C. basicorne* has a natural geographical range that extends from Spain to Azerbaijan (Alonso-Zarazaga 1990; Wanat 1995). Additional *C. basicorne* populations from elsewhere within this range may prove useful in the YST biological control program, assuming they are as host-specific as the primary population. For example, if the Turkish population does not adapt well to particular climates or to other environmental conditions after release, additional populations may be desired.

To collect morphologically identifiable, adult specimens of *C. basicorne* from field surveys and open-field host-specificity tests or other bioassays, infested plants must be uprooted and held until the adult weevils emerge from within the plant roots (Antonini et al. 2009; Balciunas and Korotyaev 2007). Field data in such experiments can be lost because of inadvertent damage to the insect within the plant during exhumation (e.g., by crushing) or because digging for test plants is done too early, in which case the uprooted plants may not remain fresh long enough for young larvae to develop to the adult stage within them (Smith et al. 2006), or because the digging is done too late, after the earliest weevils have already exited the plant (Antonini et al. 2009). Data can also be lost if weevil larvae in test plants are attacked and eaten by parasitic wasps, which are common predators of immature insects. All insects of a cohort cannot be expected to develop and eclose simultaneously since a female *C. basicorne* may oviposit in the field over the course of 1 to 2 mo (Smith et al. 2008). In cases in which multiple species are present, disparate eclosion times are even more likely. Therefore, proper timing of data collection in such experiments is critical.

Molecular biology tools should help to prevent data loss in open-field host-specificity tests, allowing researchers to collect all test plants when there is the highest probability that the test insects will simply be present within the plants, regardless of their developmental stage, rather than having to account for development and eclosion of the insects within the uprooted plants. These insects can be identified by comparison of their DNA sequence information to reference information taken from positively identified insect specimens (Antonini et al. 2008, 2009; Rauth and Hufbauer 2009; Taylor and Szalanski 1999) or gathered from publicly accessible databases, such as GenBank (<http://www.ncbi.nlm.nih.gov/>).

The purpose of this experiment was to examine the host-specificity of a *C. basicorne* population from southern France that may be useful in biological control of YST, in the event that supplemental populations are needed for the Turkish populations that are being developed as candidate biocontrol agents. This study also sought to demonstrate the utility of integrating molecular tools with traditional techniques for morphological insect identification to reduce the workload of taxonomic specialists and to increase data yield from a naturally infested, open-field host-specificity test in which several congeneric insect species were expected to be present.

Materials and Methods

Open-Field Host-Specificity Test. A field test under natural infestation conditions was conducted in southern France within the reported native ranges of several *Ceratapion* spp. known to develop in host plants of the tribe Cardueae, including *C. basicorne* (Alonso-Zarazaga 1990; Wanat 1995). Eight Cardueae species were included in the test, including YST, American star-thistle (*Centaurea americana* Nutt.), cornflower (*Centaurea cyanus* L.), Italian thistle (*Carduus pycnocephalus* L.), bull thistle [*Cirsium vulgare* (Savi) Ten.], Scotch thistle (*Onopordum acanthium* L.), globe artichoke (*Cynara scolymus* L.), and safflower (*Carthamus tinctorius* L.). A ninth Asteraceae species, garden lettuce (*Lactuca sativa* L.), was also included. Four of the test plant species (YST, *C. pycnocephalus*, *C. vulgare*, and *O. acanthium*) were known to be host plants of some of the *Ceratapion* spp. reported from southern France (Alonso-Zarazaga 1990; Wanat 1995; see Table 1). The other test plant species included two congeners of the target weed, one of which, *C. americana*, is native to the United States and sympatric with YST in part of its invaded range. The other congener, *C. cyanus*, is an ornamental flower (cornflower, also called bachelor's button) that is a known host of *C. basicorne* (Balciunas and Korotyaev 2007; Smith 2007; Smith et al. 2008). The remaining test plant species were agricultural crops; two varieties of safflower were included in the study because this species was thought to be

Table 1. List of *Ceratapion* spp. from which DNA was extracted from positive, morphologically identified, adult specimens for use as reference sequences in this study. Recorded host plants of each species are provided. Table adapted from Antonini et al (2009), based on data from Alonso-Zarazaga (1990) and Wanat (1995).

<i>Ceratapion</i> species	Host plants
<i>C. basicorne</i>	<i>Centaurea solstitialis</i> , <i>Centaurea cyanus</i>
<i>C. damryi</i>	<i>Carthamus</i> , <i>Cynara</i> (including artichoke)
<i>C. gibbirostre</i>	<i>Carduus</i> , <i>Cirsium</i>
<i>C. onopordi</i>	<i>Carduus</i> , <i>Centaurea</i> , and others
<i>C. orientale</i>	<i>Centaurea solstitialis</i> , <i>C. cyanus</i> , and others
<i>C. penetrans</i>	<i>Centaurea maculosa</i> , <i>C. cyanus</i> , and others
<i>C. scalptum</i>	<i>Silybum</i> , <i>Carthamus</i> (including safflower)

able to support *C. basicorne*, based on preliminary laboratory data (since substantiated: Smith 2007; Smith et al. 2006, 2008).

Rosettes of four test plant species—YST, *C. pycnocephalus*, *C. vulgare*, and *O. acanthium*—were uprooted from local field populations in the winter of 2003, potted, and grown outdoors at the European Biological Control Laboratory (EBCL; Montpellier, France) until the field test site was ready in the spring. Four other test species, *C. americana*, *C. cyanus*, *Lactuca sativa*, and the two safflower varieties ('Oleico' and 'Linoleico'), were grown from seed in a growth chamber in the winter of 2003, transplanted to larger pots, and moved outdoors once their root systems had established sufficiently. Potted, organically grown, artichoke rosettes were obtained from a local nurseryman shortly before the start of the field test. To mitigate exposure of the potted plants to the elements, they were occasionally taken into an unheated greenhouse to avoid hard frosts.

A field site was established in a sheep pasture in Viols-le-Fort, France. This pasture was known to have a large population of YST that supported a large population of *C. basicorne*. (During the previous spring [2002], 15 of 22 YST rosettes [68%] uprooted at random from throughout this pasture contained *C. basicorne* or had root damage typical of *C. basicorne* infestation.) A fenced enclosure (10 m × 20 m [33 ft × 66 ft]) was established in the pasture to exclude sheep or wild boar. Within the boundaries of the enclosure, 500 holes of ~20 cm (~8 in) in diameter were dug using a motorized auger and pickaxes. The characteristics of the test plot were considered sufficiently uniform to preclude blocking and on March 27, 2003, 50 rosettes of each test genotype were transplanted into the holes in a completely randomized design. Test plants were transplanted into the plot to avoid any bias on the part of the insects for transplanted vs. existing plants, to ensure that all test plants were at a similar growth stage when encountered by the insects, and to be able to evenly space the plants and

arrange them in the experimental design. Test plants were spaced 40 to 50 cm from each other and were identified by small, plastic stakes. They were watered every 3 to 10 d, depending on the weather. All YST, *C. pycnocephalus*, *C. vulgare*, and *O. acanthium* plants within the test enclosure that were not transplanted within the enclosure were removed.

Rosettes of YST outside the enclosure, both at the foot of the fence and farther afield, were periodically uprooted at random during the test period and dissected to observe the life stages of *C. basicorne* present in the field. Data were collected from the test plot on June 4 and 5, 2003, when approximately one-half of the *C. basicorne* found in the YST roots outside the test area were either pupae or newly eclosed adults. Test plants were uprooted, dissected, and examined for signs of weevil damage. Any insect found within a test plant root or crown was immediately collected into 95% ethanol in a 2-ml tube marked with the serial number of the test plant. Signs of weevil damage, without weevils present, were noted (i.e., typical weevil galleries with frass, exit holes, or parasitoids present).

Genetic Fingerprinting and Analysis. Genomic DNA was extracted from the insect specimens collected from the field experiment, as well as from expertly identified, adult specimens of *C. basicorne*, *Ceratapion scalptum* (Mulsant et Rey), *Ceratapion onopordi* (Kirby), *Ceratapion gibbirostre* (Gyllenhal), *Ceratapion orientale* (Gerstaecker), *Ceratapion penetrans* (Germar), and *Ceratapion damryi* (Desbrochers). Sequence information from *C. basicorne*, *C. onopordi*, *C. orientale*, *C. penetrans*, and *C. scalptum* were also available from a previous study (Antonini et al. 2009). The congeners of *C. basicorne* were chosen in an attempt to anticipate some of the insect species that might be collected from nontarget test plants. The selected *Ceratapion* spp. were all believed to occur in southern France and had each been recorded from at least one of the test species in the study (Alonso-Zarazaga 1990; Wanat 1995; see Table 1).

Most of the reference insect specimens were pinned and dry, although some of the *C. basicorne* adults had been stored in ethanol. Extracted DNA¹ was precipitated in ethanol, rinsed first in a solution of DNAzol : ethanol (4 : 3), followed by 70% ethanol; pelleted by centrifuge (4 min, 5,000 × g); vacuum dried; and resuspended in Tris-ethylenediaminetetraacetic acid (10 mM to 1 mM). When not in use, DNA solutions were stored at -20 C.

Fragments of the mitochondrially encoded cytochrome c oxidase I gene (MT-CO1) of each specimen were amplified by polymerase chain reaction (PCR) using the Pat and Jerry primers of Simon et al. (1994) with reagents from a commercial kit.² Previously (Antonini et al. 2009), the MT-CO1 sequence was shown to separate several *Ceratapion* spp. with a high degree of confidence. For each reaction, 2 µl of DNA solution was added to a 23-µl

Table 2. Results of Mega BLAST (National Center for Biotechnology Information) searches. All individuals identified by Mega BLAST as belonging to Apionidae were included in the subsequent analyses.

Sample	Host plant	Sampling site	GenBank accession	Mega BLAST results
Adult reference specimens				
CON2 ^a	<i>Cynara</i> sp.	Morlupo (RM), Italy	FJ621334	Brentidae; Apioninae; <i>Ceratapion onopordi</i>
COa ^a	YST	Askale, Turkey	FJ621372	Brentidae; Apioninae; <i>Ceratapion orientale</i>
CS2.1 ^a	CAUTI	Askale, Turkey	FJ621340	Brentidae; Apioninae; <i>Ceratapion scalptum</i>
CP1.6 ^a	<i>Centaurea salicifolia</i>	Krasnodar, Russia	FJ621369	Brentidae; Apioninae; <i>Ceratapion penetrans</i>
CBI6 ^a	YST	Allumiere (RM), Italy	FJ621359	Brentidae; Apioninae; <i>Ceratapion basicorne</i>
C9A	YST	Viols-le-Fort, France		Brentidae; Apioninae; <i>Ceratapion basicorne</i>
C11A ^b	YST	Viols-le-Fort, France		Brentidae; Apioninae; <i>Ceratapion penetrans</i>
Test insects				
L5M	YST	Outside fence		Brentidae; Apioninae; <i>Ceratapion basicorne</i>
L6M	YST	Outside fence		Chalcidoidea; Eulophidae; Entedoninae; <i>Horismenus missouriensis</i>
L7M	YST	Outside fence		Brentidae; Apioninae; <i>Ceratapion basicorne</i>
A8M	YST	Outside fence		Brentidae; Apioninae; <i>Ceratapion basicorne</i>
N2V	YST	50 m from test area		Brentidae; Apioninae; <i>Ceratapion basicorne</i>
N3V	YST	50 m from test area		Brentidae; Apioninae; <i>Ceratapion basicorne</i>
N4V	YST	50 m from test area		Brentidae; Apioninae; <i>Ceratapion basicorne</i>
N5V	YST	50 m from test area		Brentidae; Apioninae; <i>Ceratapion basicorne</i>
N6V	YST	50 m from test area		Brentidae; Apioninae; <i>Ceratapion basicorne</i>
N1N	CRUPY	Within test area		Brentidae; Apioninae; <i>Ceratapion scalptum</i>
N2N	CRUPY	Within test area		Brentidae; Apioninae; <i>Ceratapion scalptum</i>
N3N	CRUPY	Within test area		Brentidae; Apioninae; <i>Ceratapion scalptum</i>
L4N	CRUPY	Within test area		Curculionidae; Cryptorhynchinae; <i>Eucryptorrhynchus brandti</i>
L5N	CRUPY	Within test area		Brentidae; Apioninae; <i>Ceratapion scalptum</i>
L6N	CRUPY	Within test area		Brentidae; Apioninae; <i>Ceratapion scalptum</i>
N7N	CRUPY	Within test area		Brentidae; Apioninae; <i>Ceratapion scalptum</i>
N8N	CRUPY	Within test area		Brentidae; Apioninae; <i>Ceratapion scalptum</i>
N11N	CRUPY	Within test area		Brentidae; Apioninae; <i>Ceratapion scalptum</i>
L12N	CRUPY	Within test area		Brentidae; Apioninae; <i>Ceratapion scalptum</i>
L13N	CRUPY	Within test area		Brentidae; Apioninae; <i>Ceratapion scalptum</i>
L14N	CRUPY	Within test area		Curculionidae; Cryptorhynchinae; <i>Eucryptorrhynchus brandti</i>
L15N	CYUSC	Within test area		Curculionidae; Cleoninae; <i>Rhinocyllus conicus</i>
L16N	CYUSC	Within test area		Brentidae; Apioninae; <i>Ceratapion scalptum</i>
N18N	YST	Within test area		Brentidae; Apioninae; <i>Ceratapion basicorne</i>
N19N	YST	Within test area		Brentidae; Apioninae; <i>Ceratapion basicorne</i>
L22N	YST	Within test area		Brentidae; Apioninae; <i>Ceratapion basicorne</i>
L23N	YST	Within test area		Brentidae; Apioninae; <i>Ceratapion basicorne</i>
N3B	YST	Within test area		Brentidae; Apioninae; <i>Ceratapion basicorne</i>
L5B	YST	Within test area		Brentidae; Apioninae; <i>Ceratapion basicorne</i>
L6B	CIRVU	Within test area		Brentidae; Apioninae; <i>Ceratapion scalptum</i>
N7B	CENCY	Within test area		Brentidae; Apioninae; <i>Ceratapion basicorne</i>
N8B	CENCY	Within test area		Brentidae; Apioninae; <i>Ceratapion basicorne</i>
L9B	CENCY	Within test area		Brentidae; Apioninae; <i>Ceratapion basicorne</i>
L10B	CENCY	Within test area		Brentidae; Apioninae; <i>Ceratapion basicorne</i>
L11B	CENCY	Within test area		Brentidae; Apioninae; <i>Ceratapion basicorne</i>
L12B	CENCY	Within test area		Brentidae; Apioninae; <i>Ceratapion basicorne</i>
L13B	CENCY	Within test area		Cerambycidae; Lamiinae; Agapanthiini; <i>Agapanthia cardui</i>

Table 2. Continued.

Sample	Host plant	Sampling site	GenBank accession	Mega BLAST results
L14B	CENCY	Within test area		Cerambycidae; Lamiinae; <i>Agapanthiini</i> ; <i>Agapanthia cardui</i>
L15B	CENCY	Within test area		Cerambycidae; Lamiinae; <i>Agapanthiini</i> ; <i>Agapanthia cardui</i>
L16B	CENCY	Within test area		Cerambycidae; Lamiinae; <i>Agapanthiini</i> ; <i>Agapanthia cardui</i>
L17B	CENCY	Within test area		Cerambycidae; Lamiinae; <i>Agapanthiini</i> ; <i>Agapanthia cardui</i>
L18B	CENCY	Within test area		Cerambycidae; Lamiinae; <i>Agapanthiini</i> ; <i>Agapanthia cardui</i>
L19B	CENCY	Within test area		Cerambycidae; Lamiinae; <i>Agapanthiini</i> ; <i>Agapanthia cardui</i>
L20B	CAUTI	Within test area		Cerambycidae; Lamiinae; <i>Agapanthiini</i> ; <i>Agapanthia cardui</i>
L21B	CAUTI	Within test area		Cerambycidae; Lamiinae; <i>Agapanthiini</i> ; <i>Agapanthia cardui</i>
N22B	CAUTI	Within test area		Brentidae; Apioninae; <i>Ceratapion scalptum</i>
N23B	CAUTI	Within test area		Brentidae; Apioninae; <i>Ceratapion scalptum</i>

Abbreviations: YST, yellow starthistle, *Centaurea solstitialis* L.; CRUPY, Italian thistle, *Carduus pycnocephalus* L.; CYUSC, artichoke, *Cynara scolymus* L.; CIRVU, bull thistle, *Cirsium vulgare* (Savi) Ten., CENCY, cornflower, *Centaurea cyanus* L.; CAUTI, *Carthamus tinctorius* L. 'oleico'; Sample codes beginning with A, adult; beginning with L, larva; beginning with N, pupa.

^a Sequence information from Antonini et al. 2009.

^b Morphologically identified as *Ceratapion basicorne* (Illiger).

mixture containing the following (with original concentrations given): 17.3 µl purified water, 2.5 µl reaction buffer (10×), 0.5 µl deoxyribonucleotide triphosphates (dNTP)(10 mM), 0.2 µl Taq polymerase (5 U/µl), and 1.25 µl of each primer (10 µM). The PCR amplification³ began with an initial denaturation step of 60 s at 92 C; followed by 5 cycles of 30 s at 92 C, 60 s at 48 C, and 60 s at 67 C; then 25 cycles of 30 s at 92 C, 60 s at 52 C, and 60 s at 67 C; and finally an elongation step of 7 min at 72 C.

A second set of PCR reactions was performed with chemicals from a second commercial kit,⁴ and used a modified version of the Jerry primer (5'-CAACATT-TATTTTGATTCTTTGG-3') along with the previously cited Pat oligonucleotide. An aliquot of the genomic DNA suspension was used as template for each PCR reaction, and therefore, 2 µl of DNA solution was added to a 48 µl mixture containing the following (with original concentrations given): 29.6 µl purified water, 5.0 µl reaction buffer (10×), 4.0 µl MgCl₂, 5.0 µl dNTPs (10 mM), 2.0 µl of each primer (10 pmol µl⁻¹), and 0.4 µl Taq polymerase (5 U µl⁻¹). These amplifications were performed with the following cycling conditions: initial denaturation at 94 C for 5 min, followed by 35 cycles of denaturation at 94 C for 60 s, annealing at 50 C for 30 s, 60 s extension at 72 C; and a final elongation step of 7 min at 72 C. All PCR products were enzymatically purified using a commercial

kit⁵ and sent for sequencing to an external sequencing service,⁶ mixed with the modified version of the Jerry primer.

All gathered sequences were screened by running a blast search over the National Center for Biotechnology Information (NCBI) GenBank nucleotide collection using the Mega BLAST algorithm (Wheeler et al. 2007) available at its website (<http://www.ncbi.nlm.nih.gov/blast>). The screening procedure first assigned sequences to high-level taxa (e.g., family and subfamily), allowing removal from subsequent analyses of sequences obtained from insects outside of our interest (e.g., Hymenoptera: Eulophidae and Coleoptera: Cerambycidae; see Table 2).

Scored sequences were edited and aligned using the Staden Package⁷ software (Bonfield et al. 2006; Staden et al. 2000). All peaks were checked for wrong base calls and noise and were cleaned when required; the alignment was visually assessed without requiring any insertion-deletion (indel) typing. Divergence analyses and neighbor-joining (NJ)(Saitou and Nei 1987) tree inference were performed by means of Molecular Evolutionary Genetics Analysis, version 4 (MEGA4),⁸ setting the P-uncorrected model for the genetic distance values computation (Tamura et al. 2007); the standard errors were estimated by bootstrap method using 1,000 computation replicates. Distances were computed as pairwise values among all tested

Table 3. Computed P distances between clusters observed on the scored trees (see Figure 1). Distances were computed in MEGA4 as the net average distances between groups of taxa, given by $dA = dXY - (dX + dY)/2$, where, dXY is the average distance between groups X and Y , and dX and dY are the mean within-group distances. Values in parentheses are standard errors; in the among-groups matrix, standard errors in the upper matrix correspond to reciprocal cells' P distances in the lower matrix.

	Within groups	Among groups				
		1	2	3	CON2	COa
		P distance (SE)				
Group 1	0.003 (0.001)		(0.013)	(0.013)	(0.015)	(0.016)
Group 2	0.002 (0.002)	0.113		(0.013)	(0.015)	(0.014)
Group 3	0.007 (0.001)	0.114	0.105		(0.013)	(0.013)
CON2	—	0.144	0.138	0.116		(0.014)
Coa	—	0.155	0.136	0.118	0.127	
Group 4	0.153 (0.012)	0.162	0.144	0.148	0.141	0.136

Abbreviations: CON2, *Ceratapion onopordi*; COa, *Ceratapion orientale*.

individuals (data not shown) and as net averages among the groups that were scored on the inferred NJ topology (Table 3). Confidence at tree nodes was determined by bootstrapping 1,000 times over the data.

A Bayesian analysis was performed using MrBayes software, version 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) under the (generalized time-reversible [GTR] + I + G) substitution model selected by Akaike's Information Criterion (AIC) in MrModeltest 2.3 (Nylander 2004). The analysis was carried out using random starting trees and running 3.0×10^6 generations with Markov chains sampled every 1,000 generations. To ensure sampling of topologies after chain convergence, we discarded the first 1,000 trees as burn-in. The remaining trees were combined into a 50% majority-rule consensus tree; the percentage of samples recovering a given clade reflects the posterior probability of the clade.

Results and Discussion

Most of the field-collected insect specimens from uprooted plants (including test plants and plants dug from the field outside the test) yielded DNA that was suitable to produce sequence data via PCR amplification (95%; $n = 78$). In similar studies conducted by Antonini et al. (2008), 100% ($n = 75$) of field-collected insects provided useful data. The high yield of useful data realized through integration of molecular and morphological techniques in species identification provides meaningful justification for the use of this strategy compared with data collected by traditional means. For example, in a YST field test conducted in Turkey by Smith et al. (2006), as many as 91% of insect specimens collected at a given location in a given year were unidentifiable. Over 3 yr of testing, 11 of 30 insects (37%) collected from a key nontarget plant species (viz safflower) were unidentifiable by traditional means.

The results of the field test are shown in Table 4. The main purpose of a host-specificity test is to determine whether the biological control candidate in question will attack nontarget species. Therefore, it was essential to determine whether any insects observed attacking nontarget plants were *C. basicorne*. Of the nine plant species tested, only lettuce and Scotch thistle were without any weevils or apparent weevil damage (see Table 4; N.B., the Oleico variety of safflower was attacked, whereas the Linoleico variety was not). Of the 487 plants that were uprooted in this test, 32 contained curculiform insects, including 20 nontarget plants. However, while almost all of the field-collected insects in this experiment provided useful data through the use of molecular tools, it still appears that there were more missing data within the test (viz the number of parasitoids plus the number of plants with typical weevil damage but no weevil; see Table 4) than there were field-collected insects. Forty-two plants, including 37 nontargets, either contained parasitoids or had galleries typical of weevil infestation but without any insects present upon data collection. In the case of parasitoids, it might be possible to infer the species of weevil that had been present if the parasitoid species collected were known to be host-specific. Indeed, identification of the parasitoid species could also be facilitated with a morphological-molecular identification approach similar to that used here to identify the weevils (Taylor and Szalanski 1999). Observation of weevil damage without a weevil present is presumed to be due to the precocious development and emergence of the adult weevil (or its parasitoid) because the legless larvae of *Ceratapion* spp. are not known to leave their host plant before pupation. In such cases, it may be possible to infer the species of weevil that had been present if a molecular genetic analysis of frass or of other remnants of the former gallery occupant can be performed (Fumanal et al. 2005). Neither frass nor parasitoid remains were analyzed in this study. Therefore, the 42 plants with parasitoids or empty

Table 4. Results of open-field host-specificity test for *Ceratapion basicorne* showing the number of plants infested with curculiform insects for each plant species tested.

Plant species	Total	No. of plants							
		with insects	with larvae	with pupae	with adults	with >1 insect	with typical feeding and parasitoids	with typical feeding but no insects	Other insects present?
<i>Centaurea solstitialis</i>	50	12	5	5	2	2	2	3	N
Artichoke	49	1	1	0	0	0	0	1	Y
Lettuce	49	0	0	0	0	0	0	0	Y
Safflower (linoleic)	50	0	0	0	0	0	0	0	N
Safflower (oleic)	48	2	0	2	0	0	0	0	N
<i>Carduus pycnocephalus</i>	49	7	2	4	1	3	4	14	Y
<i>Centaurea americana</i>	44	0	0	0	0	0	0	2	N
<i>Centaurea cyanus</i>	50	7	4	3	0	0	1	4	Y
<i>Cirsium vulgare</i>	50	3	1	0	2	1	1	10	Y
<i>Onopordum acanthium</i>	48	0	0	0	0	0	0	0	N

galleries represent missing data typical of a traditional experiment.

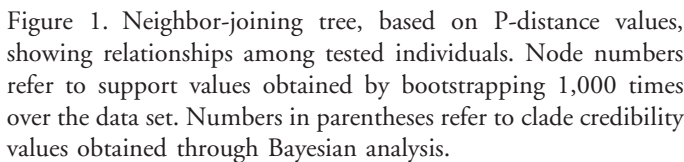
Data were gathered from this experiment after periodic surveys outside the test area revealed that approximately half of the weevils infesting YST plants were either pupae or adults (data not shown), as is typically done in a traditional experiment (e.g., Uygur et al. 2005). This timing seeks to minimize the amount of time necessary for immature weevils to complete their development within uprooted plants. This approach is balanced against the risk that data collection will occur after the emergence of the earliest adults. Such timing is imprecise, and data loss from adult emergence may be exacerbated by inclement weather conditions that delay fieldwork (e.g., digging up test plants) or by exceptionally warm weather that accelerates larval and pupal development.

Under the integrated morphological–molecular paradigm, researchers have more control over when and in which life-stage to collect test insects, since there is no need to rear the collected immature insects to adulthood. Thus, in the present study, it would have been preferable to uproot the test plants and collect insects when the first *C. basicorne* pupae were observed in YST plants outside the test plot. This would have minimized the loss of data due to precocious adult weevil emergence. In addition, since the period from oviposition of the first eggs through egg hatching and larval development for *C. basicorne* is longer (~2.5 mo [Smith et al. 2008]) than the oviposition period of overwintered adult females (1 to 2 mo [Smith and Drew 2006]), the loss of data due to digging up the plants before all weevil eggs had been laid would also have been minimized. Digging at an earlier date might also have salvaged data from some of the parasitoid-attacked weevils, which may have been recovered before being fully consumed by the parasitoids.

Amplification and sequencing of samples produced multiple sequence alignments with 39 reads of 543 nucleotide each from both primer combinations. Most of the dry, pinned reference-insect specimens did not yield DNA suitable for PCR. Therefore, five sequences from a previous study (Antonini et al. 2009) were accessed through GenBank and were included in the alignment to provide additional reference sequences from *Ceratapion* species. Those sequences were from *C. basicorne* (CBI6, GenBank FJ621359), *C. onopordi* (CON2, GenBank FJ621334), *C. orientale* (COa, GenBank FJ621372), *C. penetrans* (CP1.6, GenBank FJ621369) and *C. scalptum* (CS2.1, GenBank FJ621340).

The computed, pairwise, P-distance values ranged from 0.000 to 0.245 (data not shown), which typically cover genetic relationships between individuals belonging to the same or distinct species. The net P values computed within and among the clusters were scored on the gained topologies, taking into account intragroup variation, and are listed in Table 3 (groups 1 to 4: see Figure 1). Intracluster values ranged from 0.002 (group 2) to 0.153 (group 4), whereas values between groups ranged from 0.105 (group 2 vs. group 3) to 0.162 (group 1 vs. group 4). The latter value reflects the high intracluster variation of group 4 (0.153) due to the presence within the group of individuals likely to belong to more distantly related species or genera (L15N vs. L14N = 0.230; L15N vs. L4N = 0.230). Within-group values confirm the homogeneity of groups 1, 2, and 3, which clearly represent individual or closely related species, while also showing the heterogeneity of group 4.

The midpoint rooted topology obtained from the NJ analysis is depicted in Figure 1 (node numbers refer to the bootstrap support values). A Bayesian analysis showed virtually identical topology to the NJ tree, with five clusters



Group 2 includes CS2.1, identified as *C. scalptum* (Antonini et al. 2009), and the pupae N22B and N23B, which were collected from safflower test plants. Safflower is a known host of *C. scalptum* (Table 1) and the Mega BLAST (NCBI) search also linked these pupae to *C. scalptum* (Table 2); therefore, test insects N22B and N23B can be confidently identified as *C. scalptum*. These insects were collected from the oleic variety of safflower, whereas the linoleic variety was not attacked (Table 4).

Group 3 gathers a large number of larvae collected in *C. pycnocephalus* (L13N, N2N, N3N, N8N, L5N, N11N, N7N, L6N, L12N, N1N) and one individual collected in *Cynara scolymus* (L16N); the sample L6B was collected from *C. vulgare* and occupies a sister position to this group with a high bootstrap value. These two subgroups may represent two distinct species (average P distance between them = 0.042); however, there were no morphologically identified specimens that matched either of them. The Mega BLAST (NCBI) search linked them to *C. scalptum* (Table 2), which should be the closest relative with a sequence present in an online database. However, the tree topology clearly separates this group from group 2, which contains the *C. scalptum* reference specimen. Given their position on this tree, between *C. basicorne*, *C. penetrans*, and *C. scalptum* on one side and *C. onopordi* and *C. orientale* on the other side (Figure 1), it appears that group 3 might represent *C. dentiostre* (Gerstaecker) or *C. uniseriatum* (Faust) or both (Alonso-Zarazaga 1990; Antonini et al. 2009; Smith 2007; Wanat 1995), although neither species is represented in online sequence databases. Sequence information from *C. dentiostre* and *C. uniseriatum* would have improved the analysis of the results of this study. In addition, researchers interested in revising the genus *Ceratapion* may wish to search for specimens of these two species in roots of *Carduus pycnocephalus* and *Cirsium vulgare* near our study site.

Group 4 gathers in the most basal position the larva L15N, collected from *Cynara scolymus*, and the larvae L14N and L4N, which were collected from *C. pycnocephalus* and had identical MT-CO1 sequences. Based on their Mega BLAST (NCBI) comparisons (Table 2) and their positions on this tree (Figure 1), it appears likely that these three larvae were from outside the genus *Ceratapion*. It is noteworthy that the tree topology presented here strongly resembles, and therefore supports, the phylogenetic relationships suggested by Alonso-Zarazaga (1990) and Wanat (1995) and summarized by Smith (2007).

Within this test, 12 *C. basicorne* weevils were directly identified using this technique (six collected from YST and six from *C. cyanus*). The only other *Ceratapion* sp. that was present in the test and positively identified using the integrated strategy was *C. scalptum*, two specimens of which were collected from the Oleico variety of safflower. Although the specimens clumped in groups 3 and 4 cannot be assigned to any of the species included in this study because of a lack of reference specimens inside these clusters, it can be confidently deduced that they were not *C. basicorne*. Thus, the only nontarget plant from which specimens of *C. basicorne* were collected in this study was *C. cyanus*, an ornamental plant that has been recorded as a host of *C. basicorne* in previous studies (Balciunas and Korotyaev 2007; Smith 2007; Smith et al. 2008).

In this host-specificity test of *C. basicorne*, the type of error in species assignment that would be most significant would be a false negative, i.e., a *C. basicorne* specimen that was collected from a plant other than YST or *Centaurea cyanus* but was misidentified as a different species. The likelihood of this type of error is extremely low in this study. Specimens collected from plants other than YST and *C. cyanus* were assigned by phylogenetic analysis to groups 2, 3, and 4. Group 2 specimens were anchored to a *C. scalptum* reference specimen and were matched to *C. scalptum* by the Mega BLAST (NCBI) search. Missed *C. basicorne* specimens in group 3 would be even less likely than in group 2, given the separation in the tree topology between group 1 (containing the *C. basicorne* reference sequence) and group 3 by very high bootstrap and clade credibility values (Figure 1). Group 4 is even farther removed from group 1 and appears to contain specimens from outside the genus *Ceratapion*. In addition, to produce such a false-negative result, the Mega BLAST (NCBI) searches would need to fail to match a *C. basicorne* test specimen with the existing *C. basicorne* sequence in GenBank, which is highly unlikely when both are present (Ross et al. 2008). A false-positive result (i.e., a species other than *C. basicorne* being identified as *C. basicorne*) was not considered to be important, based on these results, because all of the test specimens identified as *C. basicorne* were found in plants already known to host this weevil. If one of them were found to be a different species, it would not affect the conclusions of the study.

Inclusion of sequence data from morphologically identified specimens of sympatric congeners of *C. basicorne*, to allow for unambiguous species identification of test specimens, illustrates the point that simple comparison of sequences from unidentified specimens with sequences (e.g., from Mega BLAST [NCBI] searches) in databases will not, in itself, provide the most reliable information. Such results are clearly biased toward whatever sequences may be deposited in the database (e.g., group 3). However, online data can be useful in providing a general idea of a test specimen's identity, as shown with several specimens collected from *C. cyanus* and safflower that matched most closely with Cerambycidae sequences, rather than with any weevil taxon (see Table 2). These specimens were clearly not weevils, much less *C. basicorne* and were, therefore, left out of the phylogenetic analysis.

Many of the dry, pinned specimens of *Ceratapion* spp. did not yield DNA suitable for PCR amplification. To avoid encountering problems of poor DNA yield, the authors highly recommend that field scientists collect at least a portion of their specimens in a manner that will allow for robust DNA extraction. DNA in a dry, pinned insect specimen degrades much faster than in a specimen preserved in alcohol or acetone (Bisanti et al. 2009; Fukatsu 1999; Hoy 1994). Even in cases in which the

species being collected is known with confidence, the presence of cryptic species (or discrete populations) with disparate host ranges or other unique characteristics is possible and may be delineated through integration of molecular techniques into the taxonomic identification, assuming sufficient sequence polymorphism can be detected with a suitable marker gene (Rauth and Hufbauer 2009).

The results presented here underscore the power of integrating morphological and molecular species identification techniques in analyzing host-specificity test data through direct inference, e.g., in the close matching of DNA sequences from test insects to those of morphologically identified specimens or to those deposited in international databases (Antonini et al. 2009), and through deduction, e.g., in using phylogenetic analysis to reject the relatedness of the biological control candidate to unidentified insects collected from nontarget test plants. This integrated approach also requires far fewer taxonomist-hours than conventional insect identification. In practical terms, there are only a limited number of expert taxonomists for any given insect taxon and therefore there may not always be one available to identify the dozens or hundreds of specimens that might be generated from a field test. In the test described here, at least four species of weevil were recovered from the test plants. Published keys exist to distinguish between some species (Balciunas and Korotyaev 2007) but their use is frequently time consuming and often technically challenging for technicians, students, or entomologists who have scant experience with the taxon in question.

On the other hand, the technical skill necessary to perform the biomolecular techniques necessary for identifications is relatively common, it is not limited to any specific taxon, and the data generated are extremely precise. In addition, once a species has been unequivocally identified by both morphological and biomolecular means, the sequence data associated with it (along with standard collection data) can be catalogued in an online database dedicated to the compilation of such data (e.g., GenBank [NCBI] or Barcode of Life Data Systems⁹ [BOLD; Ratnasingham and Hebert 2007]). As such, future studies involving these taxa may not require the assistance of an expert taxonomist. Traditional taxonomists will always be essential to classical biological control programs but with the aid of biomolecular tools for species identification, they will be able to reduce their time spent on these tasks, which are often more technical than experimental, and they will be able to provide their services to more projects in the same amount of time.

Performing host-specificity tests under open-field conditions has many advantages over laboratory or greenhouse testing (Clement and Cristofaro 1995). In addition to providing data under the most “natural” conditions

possible, naturally infested open-field tests allow more replications for less labor than contained bioassays do, particularly if the candidate biocontrol agent is difficult to rear in captivity in large numbers. Integrating molecular tools into taxonomic identification reduces the labor necessary to conduct open-field host-specificity tests because species identification represents one of the largest labor requirements for such tests. In the open-field host-specificity test reported here, an integrated technique was used to show that a French population of *C. basicorne* is worthy of further consideration as an alternate source of this biological control candidate for YST.

Sources of Materials

¹ DNAzol reagent, Invitrogen SARL, 95613 Cergy Pontoise CEDEX, France.

² Taq PCR Master Mix kit, Qiagen SA, 91974 Courtaboeuf CEDEX, France.

³ GeneAmp PCR System 2400, PerkinElmer Inc., Waltham, MA 02451.

⁴ BIOLASE PCR kit, Bioline, London NW2 6EW, United Kingdom.

⁵ ExoSAP-IT kit, USB Corporation, Cleveland, OH 44128-5933.

⁶ Sequencing performed by BMR Genomics, 35131 Padova, Italy.

⁷ Staden Package software version 1.7.0, SourceForge.net, Geeknet, Inc., Mountain View, CA 94041.

⁸ Molecular Evolutionary Genetics Analysis, version 4 (MEGA4), software, Center for Evolutionary Medicine and Informatics, The Biodesign Institute, Tempe, AZ 85287-5301.

⁹ Barcode of Life Data Systems, Biodiversity Institute of Ontario, Guelph, ON N1G 2W1, Canada.

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